STIC-ILL

From: STIC-Biotech/ChemLib
Sent: Thursday, September 06, 2001 2:20 PM
To: STIC-ILL
Subject: FW: 09/276,935 reference request

-----Original Message-----
From: Pak, Michael
Sent: Thursday, September 06, 2001 2:19 PM
To: STIC-Biotech/ChemLib
Subject: 09/276,935 reference request

Dear STIC people:

Michael D. Pak
Art Unit 1646
CM1/10E13
305-7038
09/276,935
Needed ASAP.

Please send me copies of these references.
Please also copy the table of contents page of the journal issue for each reference.

1. Kliewer et al. (1998) Cell 92: 73-82.

2. Zhang et al. (1999) Arch. Biochem. Biophys. 368: 14-22.

3. Li et al. (1997) Endocrinology 138: 2347-2353.

0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 6
Printed in U.S.A.

Cloning and Characterization of the Vitamin D Receptor from *Xenopus laevis**

YAN CHUN LI†, CLEMENS BERGWITZ, HARALD JÜPPNER, AND MARIE B. DEMAY

Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT

The Vitamin D receptor (VDR), a member of the nuclear receptor superfamily, mediates the effects of 1,25-dihydroxyvitamin D_3 on mineral ion homeostasis. Although the mammalian and avian VDRs have been extensively studied, little is known about the VDR in lower vertebrate species. To address this, we have isolated the *Xenopus laevis* VDR (xVDR) complementary DNA. Overall, the xVDR shares 79%, 73%, 73%, and 75% identity at the amino acid level with the chicken, mouse, rat, and human VDRs, respectively. The amino acid residues and subdomains important for DNA binding, hormone binding, dimerization, and transactivation are mostly conserved among all VDR species.

The xVDR polypeptide can heterodimerize with the mouse retinoid X receptor α, bind to the rat osteocalcin vitamin D response element (VDRE), and induce vitamin D-dependent transactivation in transfected mammalian cells. Northern analysis reveals two xVDR messenger RNA species of 2.2 kb and 1.8 kb in stage 60 *Xenopus* tissues. In the adult, xVDR expression is detected in many tissues including kidney, intestine, skin, and bone. During *Xenopus* development, xVDR messenger RNA first appears at developmental stage 13 (preneurulation), increasing to maximum at stages 57–61 (metamorphosis). Our data demonstrate that, in *Xenopus*, VDR expression is developmentally regulated and that the vitamin D endocrine system is highly conserved during evolution. (*Endocrinology* **138:** 2347–2353, 1997)

THE VITAMIN D endocrine system plays an important role in mineral ion homeostasis (1). 1,25-dihydroxyvitamin D_3, the active hormone, has also been found to regulate cell differentiation and cell proliferation during myogenesis and hematolymphopoiesis (2–4). The receptor that mediates the actions of 1,25-dihydroxyvitamin D_3 has been cloned from human (5), rat (6), mouse (7), and avian species (8, 9), leading to the characterization of the molecular mechanisms involved in transcriptional regulation by 1,25-dihydroxyvitamin D_3. The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily. It binds to *cis*-regulatory elements in target genes by heterodimerizing with the retinoid X receptor (RXR) and thereby regulates gene transcription (10, 11).

Although the VDRs from mammals and avians are structurally and functionally conserved (10), little is known about the VDR of lower vertebrate species such as amphibians. Amphibians are the first animals to make the water to land transition in evolution. This transition is linked to an increased dependency on dietary calcium for mineralization of a bony skeleton (12, 13) because aquatic vertebrates extract calcium mainly from the water they live in. Amphibians, therefore, represent an intermediate stage in the evolution of endocrine regulation of mineral metabolism because of their transition from an aquatic to a terrestrial environment (13, 14). To better understand the importance of the vitamin D endocrine system in calcium homeostasis from an evolutionary perspective, we cloned the *Xenopus laevis* VDR (xVDR). We also examined the expression of the VDR during development in *Xenopus laevis* because little is known about the role of the VDR in animal development. We showed that the function as well as the sequence of the VDR is well conserved in evolution and that the expression of the xVDR is developmentally regulated.

Materials and Methods

Complementary DNA (cDNA) cloning

The xVDR cDNA was cloned by RT-PCR combined with cDNA library screening. Briefly, 4 μg of *Xenopus* small intestine total RNA were reverse transcribed into first strand cDNA in a 50-μl reaction containing 1 μg of oligo-$(dT)_{12-18}$, 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs and 300 U of M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD) at 37 C for 1 h. Then 5 μl of the RT reaction were subjected to PCR in a 100-μl reaction using a GeneAmp PCR reagent kit (Perkin-Elmer, Norwalk, CT). The PCR primers used were a pair of degenerate oligonucleotides synthesized based on the conserved but least degenerate regions of the published VDR sequences. Primer 1: 5′-AT(A C T)GG(A C G T)TT(C T)GC(A C G T)AA(A G)ATGAT(A C T)CC-3′ and Primer 2: 5′-AC(A G)TG(C T)TC(C T)TC(C T)TC(C T)TC(A G)TG(A C G T)AG(A G)TT-3′ correspond to human VDR amino acid residues 242 to 250 and 324 to 331, respectively. The reaction was run for 35 cycles (94 C, 1 min; 48 C, 1 min; and 72 C 1 min). To increase the amount of the PCR product, a second round of PCR was performed under the same conditions with the exception of a higher annealing temperature (60 C). The expected 270 bp PCR product was purified and subcloned into pBluescript according to the method of Marchuk *et al.* (15). Inserts were sequenced by the dideoxynucleotide chain termination method using the Sequenase DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

For cDNA library screening, the 270-bp fragment insert was released from pBluescript with *Eco*R I and *Hin*dIII and labeled with α-^{32}P-dATP

Received January 10, 1997.

Address all correspondence and requests for reprints to: Marie B. Demay, M.D., Endocrine Unit, Wellman 501, 32 Fruit Street, Massachusetts General Hospital, Boston, Massachusetts 02114.

* This work is supported by NIH Grants DK-46974 (to M.B.D.) and P01-DK-11794. The DNA sequence reported in this paper has been deposited with Genbank (accession number U91846). A preliminary report of this work was presented in the 1996 Annual Meeting of the American Society for Bone and Mineral Research.

† NIH fellowship recipient.

(DuPont-New England Nuclear, Boston, MA) using a random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). Approximately 1×10^6 plaque forming units (pfu) from a *Xenopus* adult kidney Uni-ZAP cDNA library (Stratagene, La Jolla, CA) were plated and transferred onto Nylon membranes (NEN, Boston, MA) for hybridization. The membranes were baked for 2 h at 80 C and hybridized with the cDNA probe in 50% formamide, 6 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA at 42 C. The positive clones were plaque-purified and the inserts were released from the phage with the ExAssist helper phage according to the manufacturer's instructions (Stratagene). The sequence of both strands of the positive clones was determined as described above and compared with the previously published VDR sequences in Genbank using the GCG program.

To confirm the sequence of the xVDR cDNA clones, additional clones were isolated by PCR from a thyroid hormone-induced *Xenopus* larval tail cDNA library. The PCR primers were based on the sequence in the 5′ and 3′ untranslated regions of the first clone.

RNA isolation and Northern blot analysis

Xenopus total RNA was isolated by the guanidium-HCl/phenol extraction method (16). Poly(A$^+$) RNA was purified using an oligo-dT cellulose column as described (17). For Northern blot analysis, the poly(A$^+$) RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred onto a Nylon membrane (18). Hybridization was carried out as described above.

Ribonuclease protection assays

Riboprobes were transcribed from linearized plasmids according to the method of Krieg and Melton (19). pBluescript containing the 270 bp *Xenopus* PCR product was linearized with *Bam*HI, and the antisense riboprobe was synthesized using T7 RNA polymerase (Promega, Madison, WI) in the presence of α-^{32}P-UTP. For an internal control, a 375-bp fragment of *Xenopus* elongation factor 1 α (EF1α) in pGEM1 (20) was also transcribed and used simultaneously in the protection reactions. Somatic *Xenopus* EF1α is expressed at equal levels in all tissues and throughout development starting at mid blastula stages (20).

For RNase protection assays, 20–50 μg of total RNA (adjusted to a total amount of 100 μg with torula RNA) were coprecipitated with 0.2×10^6 cpm of xVDR riboprobe (specific activity approximately 5×10^8 cpm/μg) and 100 cpm of EF1α riboprobe (4.5×10^5 cpm/μg). The precipitates were resuspended in 30 μl of hybridization buffer (80% formamide, 40 mM PIPES (pH 6.4), 400 mM Na-Acetate, and 1 mM EDTA), denatured at 85 C for 5 min, and then incubated at 45 C for 18 h. After the hybridization, 300 μl of digestion mix containing 200 U RNase T1 (Sigma, St. Louis, MO), 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 300 mM NaCl was added, and the reaction was continued for 2 h at room temperature. The digestion was stopped by adding proteinase K to 330 μg/ml, and SDS to 0.7% and further incubating at 37 C for 15 min. The protected RNAs were then precipitated and resolved on a 5% polyacrylamide/8 M urea sequencing gel for subsequent autoradiography at −70 C for 1–8 days.

Gel retardation assays

VDR DNA binding was examined using *in vitro* synthesized proteins. xVDR, rat VDR (rVDR) and mouse RXRα (mRXRα) cDNAs were transcribed into cRNAs and translated into polypeptides using a linked transcription-translation system (Promega). Complementary oligonucleotides 5′-TGGGTGAATGAGGACAG-3′ representing the rat osteocalcin VDRE with GATC overhangs were annealed and blunt-ended with Klenow DNA polymerase in the presence of α-^{32}P-dATP. The synthesized proteins and DNA probe were incubated as described previously (21) and electrophoresed on a 6% polyacrylamide gel. The gel was dried and exposed to x-ray film (Kodak, Rochester, NY).

Cell transfection and CAT assays

COS-7 cells were grown in DMEM (Life Technologies, Gaithersburg, MD) containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 C. Cells were cotransfected by diethylaminoethyl dextran with pcDNA1 containing xVDR, rVDR, or no insert, a reporter plasmid, ID3TKCAT containing the rat osteocalcin VDRE-TKCAT (21), and RSV luciferase, as a control for transfection efficiency. Immediately after transfection, and daily thereafter, the cells were treated with 10^{-8} M 1,25-dihydroxyvitamin D_3. The cells were harvested 72 h post transfection, and cell lysates were assayed for luciferase and CAT activity (22).

Results

Cloning of the Xenopus vitamin D receptor cDNA

The xVDR cDNA was cloned by RT-PCR using degenerate primers based on the available mammalian and avian VDR sequences. To increase the PCR specificity, the sequences of the degenerate PCR primers were designed corresponding to the amino acid sequences in the hormone binding domain. The VDR hormone binding domain is conserved among mammals and avians (87.5%) (9) but differs from that of the other members of the nuclear receptor superfamily (domains E and F) (11), whereas the Zn finger DNA binding domain (domain C) (11) is conserved across the nuclear receptor superfamily members. We chose the most conserved and the least degenerate regions to minimize the degree of degeneracy of the primers. Based on the human VDR sequence, the PCR product was expected to be 270 bp. *Xenopus* small intestinal RNA was used as the source for xVDR cDNA amplification because, as in mammals and avians, amphibian small intestine is one of the primary sites for vitamin D-dependent calcium exchange (14). After the first round of PCR amplification, a faint 270-bp band was obtained; therefore, a second round of PCR was employed to reamplify this fragment with the same primers at a higher annealing temperature. To rule out the possibility of cross contamination from the control rVDR cDNA, both the *Xenopus* and the rat PCR products were hybridized with a rVDR cDNA probe at high stringency. The *Xenopus* PCR product gave a much weaker signal than the rat band in spite of equal DNA loading (data not shown), suggesting that the *Xenopus* PCR product was distinct from the rat product. Indeed, the nucleotide sequence of the *Xenopus* DNA fragment revealed 66% identity to the rVDR sequence, suggesting that this cDNA was a *Xenopus* VDR fragment.

This cDNA fragment was then used as a probe to screen a *Xenopus* adult kidney cDNA library because kidney is also a target organ for vitamin D action in *Xenopus* (14). After screening more that 10^6 pfus, only one positive clone was identified. This clone has an insert of approximately 1.8 kb and contains an open reading frame encoding a full-length *Xenopus* VDR protein. PCR products amplified from a thyroid hormone-induced *Xenopus* larval tail cDNA library also contain an identical sequence.

Analysis of the Xenopus VDR sequence

The nucleotide and deduced amino acid sequences of the xVDR cDNA clone are shown in Fig. 1. This clone is 1782 nucleotides long and has an open reading frame encoding a polypeptide of 422 amino acids, with the first Met codon located at position 141. The translational initiation sequence 5′-GTTATGG-3′ is a suboptimal version of a typical Kozak sequence (23). The 323-bp-long AT rich 3′ untranslated region contains no typical putative polyadenylation signal immediately upstream to the poly(A) tail (24). Interestingly, the 3′ untranslated sequence is much shorter than that of the

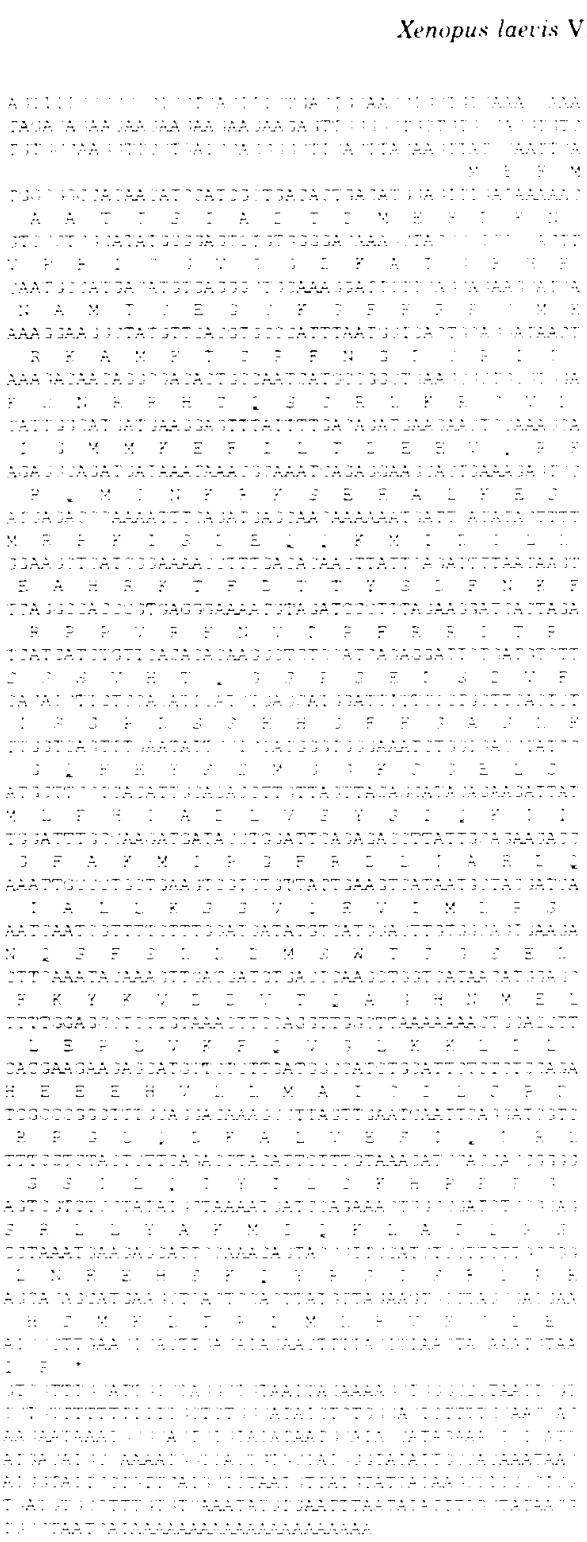

FIG. 1. The nucleotide and predicted amino acid sequences of the *Xenopus* vitamin D receptor. The nucleotides and amino acids are numbered on the *left*.

mammalian VDRs (5). The open reading frame encodes the *Xenopus* VDR polypeptide, with a calculated mol wt of 48,137 daltons, similar to that of mammalian VDRs and avian VDR form B, and smaller than avian form A that arises from an alternate translational initiation site (9).

Figure 2 shows the alignment of the human (h), rat (r), mouse (m), chicken (c) (form B), and xVDR amino acid sequences. Overall the xVDR shares 75%, 73%, 73%, and 79% identity, at the amino acid level, with the human, rat, mouse, and chicken VDRs, respectively (Table 1). The similarity among them is even higher (Table 1). Table 2 compares the functional domains of the hVDR to the VDRs of other species. In the DNA binding domain [amino acids 22 to 114, (10)], the xVDR is 93% identical to the hVDR, whereas the rat, mouse, and chicken VDRs are 99%, 99%, and 97% identical, respectively. The eight cysteine residues, critical for Zn finger formation, are completely conserved (Fig. 2). In the hormone binding domain (amino acid 196 to the C-terminus), the xVDR is only 76% identical to the hVDR, whereas the rat, mouse, and chicken VDRs are 91%, 90%, and 83% identical, respectively. More importantly, the regions or amino acid residues in this domain so far identified to be vital for 1,25-dihydroxyvitamin D_3 binding, heterodimerization with RXR and transactivation are mostly conserved across all the species (see discussion). The hinge region between these two functional domains is more divergent, with the xVDR sharing only 45% identity with the hVDR and 63% with the cVDR.

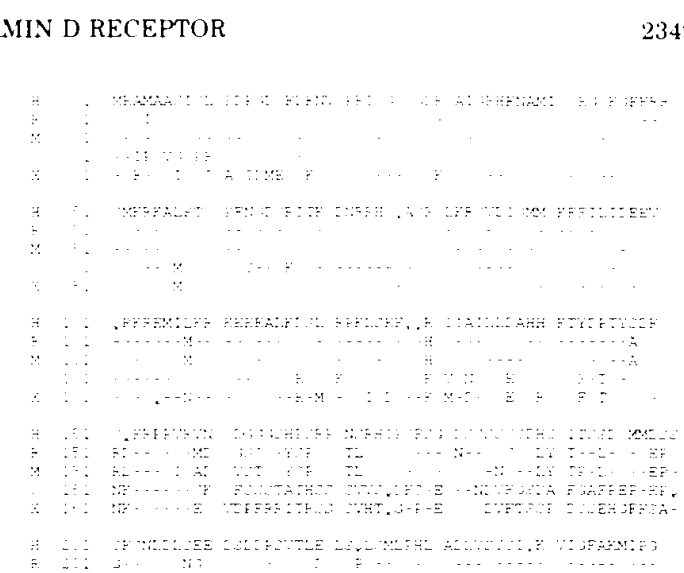

FIG. 2. The alignment of the human (H), rat (R), mouse (M), chicken (C), and *Xenopus* (X) VDR amino acid sequences. Sequences were best aligned to each other and to the hVDR. Gaps indicate the absence of amino acids. The amino acids that differ from those of the hVDR are shown in the other species. Only the B form of the cVDR is shown (9).

TABLE 1. Comparison of the *Xenopus* VDR amino acid sequence with that of other species

Species	Similarity	Identity
Human	87.5%	74.5%
Rat	83.6%	72.9%
Mouse	84.3%	72.9%
Chicken	87.9%	78.8%

TABLE 2. Comparison of human VDR functional domains with those of other species

Species	DBD	HR	HBD
Rat	99%	75%	91%
Mouse	99%	74%	90%
Chicken	97%	44%	83%
Xenopus	93%	45%	76%

DBD, DNA-binding domain; HR, hinge region; HBD, hormone-binding domain.

Functional characterization of xVDR

The identity of the cloned xVDR was confirmed by gel retardation assays and transient gene expression experiments. To examine the VDR-VDRE interactions, *in vitro* synthesized mRXRα, rVDR, and xVDR were used. As shown in the first three lanes of Fig. 3, neither the mRXRα, the rVDR, nor the xVDR alone binds to the probe (RXR, rVDR, xVDR). Like the rVDR, the xVDR can dimerize with the mRXRα and bind to the rat osteocalcin VDRE (shown in the following lanes). The binding is competed for by excess unlabeled probe. The xVDR-mRXRα heterodimer generates a slower migrating and less intense band than that seen with the rVDR-mRXRα heterodimer. This phenomenon was observed in several repeated experiments. The difference in the intensity and mobility of the protein-DNA complex may reflect the difference in DNA-protein or protein-protein interactions due to species differences; or a difference in the conformation of the xVDR-mRXRα complex because the size of the two VDR proteins is similar. (The xVDR is one amino acid shorter).

The function of the xVDR was examined by transfection experiments. When COS-7 cells were transiently cotransfected with both the xVDR and a VDRE-TK-CAT fusion gene, CAT activity was induced 2.3-fold in response to 10^{-8} M 1,25-dihydroxyvitamin D_3 treatment. Under the same conditions, the rVDR mediated 6-fold induction, and the control plasmid pcDNA1 showed no induction (Fig. 4). The weaker induction mediated by the xVDR may be due to impaired interactions with mammalian RXRα, or decreased affinity for mammalian VDRE as suggested by the gel retardation assays. The data from these two assays support the identity of this clone as a functional VDR.

xVDR messenger RNA (mRNA) and its tissue distribution

Two forms of xVDR mRNA were detected in Northern blot analysis of developmental stage 60 *Xenopus* (Fig. 5). The mRNA sizes of about 1.8 kb and 2.2 kb are smaller than the mammalian VDR mRNA (~4.6 kb). Two VDR mRNA species (2.6 kb and 3.2 kb) were previously reported in chicken intestine and kidney (8).

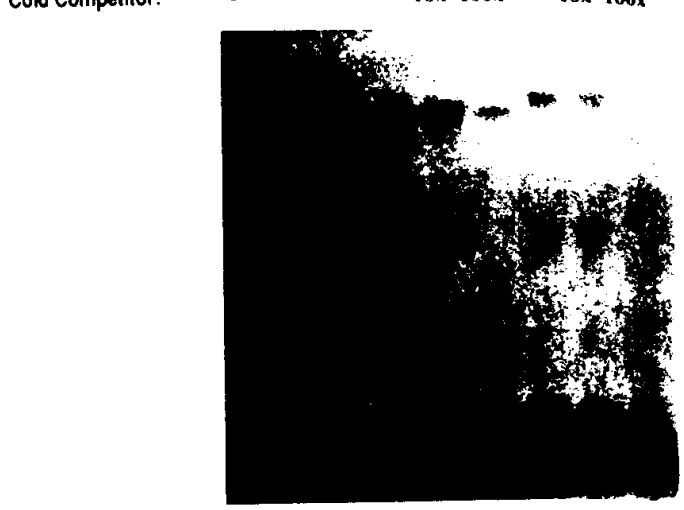

FIG. 3. Binding of the xVDR to the rat osteocalcin VDRE. Gel retardation assays were performed using *in vitro* translated mRXRα, rVDR and xVDR. A ^{32}P-labeled oligonucleotide containing the rat osteocalcin VDRE was used as a probe. The first three lanes contain mRXRα, rVDR, and xVDR alone. The mRXRα and rVDR, together with the probe, generate a retarded complex that is competed for by 10- and 100- fold excess of the unlabeled probe. A fainter, slower migrating complex with similar competition is generated by the mRXRα, xVDR and probe.

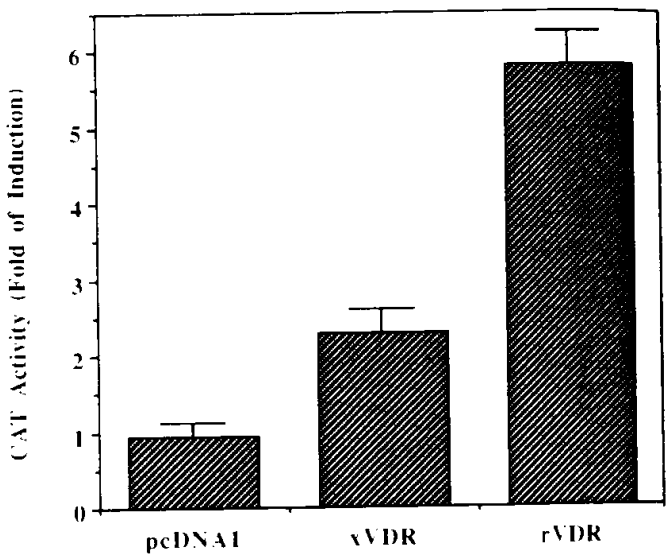

FIG. 4. xVDR-mediated 1,25-dihydroxyvitamin D_3-dependent transactivation of CAT activity in transfected cells. COS-7 cells were cotransfected with a VDRE-CAT fusion gene, RSV luciferase and rVDR, xVDR or control plasmid (pcDNA1). The cells were treated with or without 10^{-8} M 1,25-dihydroxyvitamin D_3 for 72 h. The CAT activity was normalized for luciferase activity and is presented as fold of induction by 1,25-dihydroxyvitamin D_3 treatment. The data and SEM are derived from four independent experiments.

The tissue distribution of the xVDR was assessed by RNase protection assay (Fig. 6). xVDR mRNA was detected in all the tissue examined, including kidney, lung, heart, liver, brain, small intestine, ovary, skeletal muscle, skin, and bone. Small intestine and skin express the highest level of xVDR mRNA.

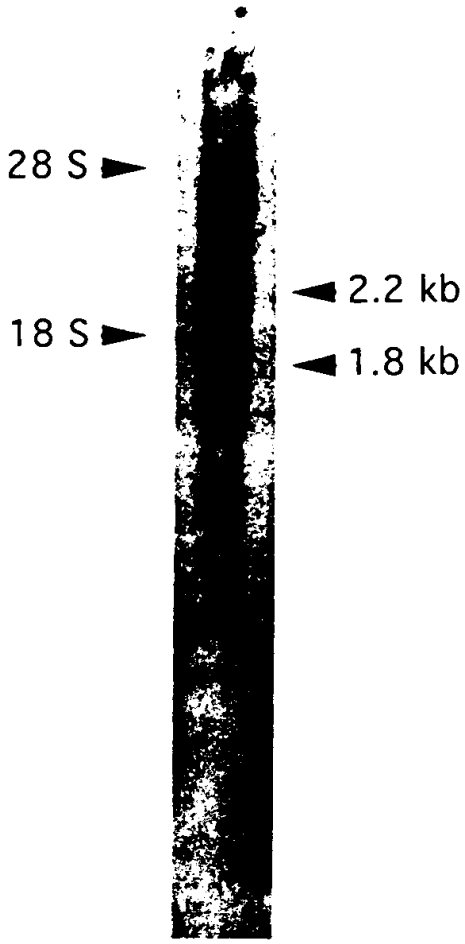

FIG. 5. Northern blot analysis of xVDR mRNA. Poly(A$^+$) RNA (5 μg) purified from 500 μg of *Xenopus* stage 60 total RNA was probed with the xVDR cDNA. As indicated, two sizes of xVDR mRNA are detected.

Regulation of xVDR expression in Xenopus development

xVDR expression was further studied at different stages during *Xenopus laevis* development. In the RNase protection assay shown in Fig. 7, xVDR mRNA was first detected at the early neurula stage (stage 13), the time of neural plate development (25). The mRNA level gradually increases during *Xenopus* development and peaks at stages 57 to 61, when metamorphosis is taking place (25). The postmetamorphotic mRNA levels then decrease to the level seen in the adult toad.

Discussion

Amphibians occupy a pivotal place in evolution. They are the first animals to make the water to land transition, bridging the evolutionary gap between aquatic and terrestrial vertebrates. The transition from an aquatic to a terrestrial environment requires changes in the mechanism controlling calcium homeostasis (13, 14). The aquatic animals (fish and tadpoles) obtain calcium through their gills and skin from an

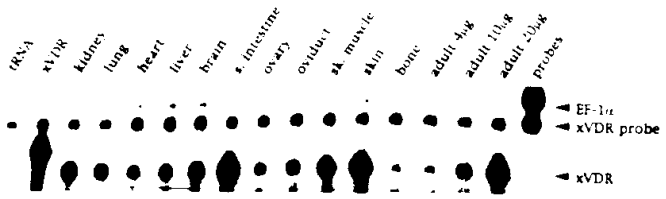

FIG. 6. The tissue distribution of xVDR mRNA. Total RNA was isolated from each of the tissues indicated and from the whole animal (adult). For each tissue, 40 μg of total RNA were subjected to RNase protection to examine the relative abundance of xVDR mRNA. *Xenopus* EF-1α serves as an internal control for the amount of RNA used.

inexhaustible reservoir of dissolved mineral ion in the water (26–28), whereas the terrestrial tetrapods depend mainly on dietary calcium (12). The high level of VDR mRNA in *Xenopus* skin relative to bone may reflect the importance of the skin in calcium homeostasis in this primarily aquatic amphibian. In addition to bones, some amphibian species have specialized endolymphatic sacs for calcium storage. This calcium can be mobilized for ossification of the cartilaginous skeleton during metamorphosis (29, 30). Calcium metabolism in amphibians is regulated by PTH, calcitonin, and vitamin D as well as by PRL and stanniocalcin (13, 14, 31).

As in the higher vertebrates, the primary function of vitamin D in amphibian calcium metabolism seems to be raising blood calcium (12, 13), however, the relative importance of the various target organs differs. Vitamin D-dependent calcium absorption and reabsorption in amphibian intestine and kidney have been reported (32, 33), and calcium-binding protein (CaBP) has been found in these organs (33, 34). Vitamin D treatment enhances calcium uptake by skin (26) and calcium accumulation in the endolymphatic sacs (35, 36). Furthermore, vitamin D has been shown to play a critical role in amphibian skeletal development. When maintained on vitamin D-deficient diets, *Xenopus laevis* develops skeletal abnormalities (rickets and osteoporosis) (37). These data support the hypothesis that the vitamin D endocrine system appears early in phylogeny and is functionally conserved during evolution. Indeed, our data presented in this paper indicate that the sequence of the vitamin D receptor is well conserved from *Xenopus* to mammals. The observation that the xVDR dimerizes with the mRXRα and binds to a rat osteocalcin VDRE to confer 1,25-dihydroxyvitamin D_3-dependent transactivation further confirms the functional conservation of the receptor. Interestingly, the receptor for PTH/PTHrP, two hormones intimately involved in calcium homeostasis, is also conserved during evolution. The xPTH/PTHrP receptor isoforms share 69–78% identity with the mammalian receptors (Bergwitz, C., P. Klein, J. Graff, H. Kohno, S. Forman, D. Rubin, K. Lee, G. V. Segre, D. Melton, and H. Jüppner, manuscript submitted).

Like other members of the nuclear receptor superfamily, the VDR contains an N-terminal DNA-binding domain, which interacts with the VDRE through its two Zn fingers, and a C-terminal ligand-binding domain, which is responsible for high affinity 1,25-dihydroxyvitamin D_3 binding, dimerization, and transcriptional activation (10). As shown in Fig. 2, among all VDR species examined, the eight cysteine residues vital for the Zn finger formation, and other residues

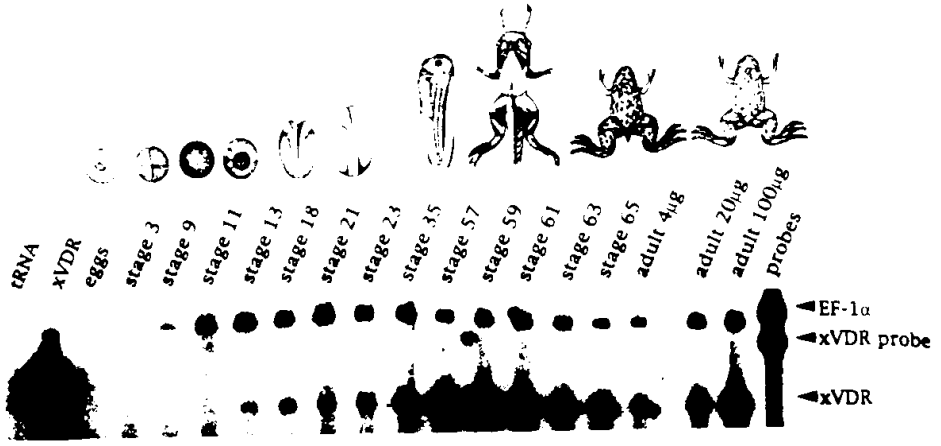

FIG. 7. xVDR expression during development. The expression of xVDR mRNA was examined by RNase protection assay at various stages of *Xenopus* development, using 20 μg of total RNA per reaction. *Xenopus* EF1α serves as an internal control. The major morphological changes associated with some developmental stages examined are presented in the *top panel*.

forming the Zn finger structure, are conserved. The ligand-binding domain, like that of other nuclear receptors, contains nine heptad repeats thought to constitute a dimerization motif (39), and an E1 region near the N-terminal boundary believed to be crucial for receptor function (40). Studies of the hVDR function using site-directed mutagenesis and examining naturally occurring mutations revealed that heptad 4 and 9 (41), R391 (42), and the E1 region (specifically F244, K246, L254, Q259 and L262) (40), are essential for heterodimerization. Furthermore, these hVDR studies also showed that R274 (43), C288, C337 (44), E395, H397 and K399 (41) are important for high affinity binding of 1,25-dihydroxyvitamin D_3. All these residues and subdomains are conserved from amphibian to mammalian VDRs (Fig. 2). These data strongly argue that the VDR is well conserved throughout evolution.

Residue I314 of the hVDR was found to be involved in ligand-mediated transactivation in a study of a mutated VDR from a patient with hereditary hypocalcemic vitamin D-resistant rickets (42). When I314 is mutated into S314, VDR hormone responsiveness, heterodimerization, and transcriptional activation are impaired (42). Interestingly, I314 is conserved in all mammalian VDRs but not in c- and xVDRs, where the Ile is replaced with a Val. It is possible that the Val substitution at this position will diminish VDR dimerization and transactivation. This substitution may help to explain the decreased intensity of the protein-DNA complex in gel retardation assays and the decreased ligand-dependent transactivation observed with the xVDR in COS cells.

The xVDR mRNA is detected in a broad range of tissues in *Xenopus laevis*. In addition to the traditional target organs such as intestine, kidney, bone and skin, xVDR mRNA was also seen in heart, lung, liver, brain, ovary, oviduct, and muscle. At least in chicken and rat, no VDR mRNA was detected in the liver, and very low levels were found in the heart and oviduct (6, 8). The function of the xVDR in these nontraditional target tissues is unclear. It is possible that the xVDR may play different roles in amphibians and in higher vertebrates.

Nuclear receptor-mediated hormone actions have been shown to be critical for *Xenopus laevis* development. Retinoids regulate anterior-posterior axis formation of *Xenopus* embryos (45–49). Consistent with this observation, high level expression of the retinoic acid and retinoid X receptors are detected at early stages (from oogenesis to gastrulation, stage 7–8) of *Xenopus* development (50, 51). Thyroid hormone acts as a biological effector of amphibian metamorphosis and limb development. *Xenopus* metamorphosis is characterized by profound morphological changes including the development of limbs, ossification of the cartilaginous skeleton, and resorption of the tail (25). At this time, expression of the *Xenopus* thyroid hormone receptor reaches its maximum, correlating with the peak secretion of thyroid hormone (52). We first detected the xVDR mRNA at early neurulation (stage 13), almost immediately after the sharp decline of retinoic acid and retinoid X receptor levels (51). From this point on, the mRNA level steadily increases as development continues, reaching maximum at metamorphosis. Although the level of 1,25-dihydroxyvitamin D_3 has not been reported, the plasma calcium concentration is markedly increased at the time of metamorphosis (14), providing the minerals needed for skeletal ossification. Once the animal is morphologically an adult and its skeleton is mineralized, the requirement for the receptor-dependent action of 1,25-dihydroxyvitamin D_3 is not as great, and the VDR mRNA levels decrease to the adult steady state level. These data suggest that the xVDR is also critical for *Xenopus* metamorphosis, and the appearance of the xVDR in the early *Xenopus* embryo, before the development of a bony skeleton, implies that the function of the xVDR extends beyond the regulation of calcium homeostasis.

Acknowledgment

We thank Alison Pirro, Wendy Simays, and Jennifer Heymont for technical supports, Dr. Henry Kronenberg for critical reading of the manuscript

References

1. **DeLuca HF** 1988 The vitamin D story: a collaborative effort of basic science and clinical medicine. FASEB J 2:224–236
2. **Manolagas SC, Yu X-P, Girasole G, Bellido T** 1994 Vitamin D and the hematolymphopoietic tissue: a 1994 update. Semin Nephrol 14:129–143
3. **Liu M, Lee M-H, Cohen M, Bommakanti M, Freedman LP** 1996 Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 10:142–153
4. **O'Connell TD, Giacherio DA, Jarvis AK, Simpson RU** 1995 Inhibition of cardiac myocyte maturation by 1,25-dihydroxyvitamin D3. Endocrinology 136:482–488
5. **Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW** 1988 Cloning and expression of full-

length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 85:3294–3298

Burmester JK, Wiese RJ, Maeda N, DeLuca HF 1988 Structure and regulation of the rat 1,25-dihydroxyvitamin D3 receptor. Proc Natl Acad Sci USA 85:9499–9502

Kamei Y, Kawada T, Fukuwatari T, Ono T, Kato S, Sugimoto E 1995 Cloning and sequencing of the gene encoding the mouse vitamin D receptor. Gene 152:281–282

McDonnell DP, Mangelsdorf DJ, Pike JW, Haussler MR, O'Malley BW 1987 Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. Science 235:1214–1217

Elaroussi MA, Prahl JM, DeLuca HF 1994 The avian vitamin D receptors: primary structures and their origins. Proc Natl Acad Sci USA 91:11596–11600

MacDonald PN, Dowd DR, Haussler MR 1994 New insight into the structure and functions of the vitamin D receptor. Semin Nephrol 14:101–118

Tsai M-J, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486

Robertson DR 1989 Regulation of calcium and phosphate by intestine. In: Pang PKT Schreibman MP (eds) Vertebrate Endocrinology: Fundamentals and Biomedical Implications. Academic Press, San Diego, pp 243–275

Pandey AK 1992 Endocrinology of calcium metabolism in amphibians, with emphasis on the evolution of hypercalcemic regulation in tetrapods. Biol Struct Morphog 4:102–126

Stiffler DF 1993 Amphibian calcium metabolism. J Exp Biol 184:47–61

Marchuk D, Drumm M, Saulino A, Collins FS 1991 Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res 19:1154

Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Sambrook J, Fritsch EF, Maniatis M 1989 Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Ausubel FM, Brent R, Kinston RE, Moore DD, Seidman JG, Smith JA, Struhl K 1984 Current protocols in molecular biology. John Wiley & Sons, New York, NY

Krieg PA, Melton DA 1984 Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned DNAs. Nucleic Acids Res 12:7057–7070

Krieg PA, Varnum SM, Wormington WM, Melton DA 1989 The mRNA encoding elongation factor 1 alpha (EF1α) is a major transcript at the mid-blastula transition in Xenopus. Dev Biol 133:93–100

Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM 1990 DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci USA 87:369–373

Goto K, Heymont JL, Klein-Nulend J, Kronenberg HM, Demay MB 1996 Identification of an osteoblastic silencer element in the first intron of the rat osteocalcin gene. Biochemistry 35:11005–11011

Kozak M 1986 Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283–292

Wickens M, Stephenson P 1984 Role of the conserved AAUAAA sequence: four AAUAA point mutants prevent messenger RNA 3' end formation. Science 226:1045–1051

Nieuwkoop PD, Faber J 1975 Normal table of *Xenopus laevis* (Daudin). A systematical and chronological survey of the development from the fertilized egg till the end of metamophosis. North-Holland Publishing Company, Amsterdam

Baldwin FM, Bentley PJ 1980 Calcium metabolism in bullfrog tadpoles (*Rana catesbeiana*). J Exp Biol 88:357–365

Watlington CO, Burke PK, Estep HL 1968 Calcium flux in isolated frog skin: the effect of parathyroid substances. Proc Soc Exp Biol Med 128:853–856

Baldwin GF, Bentley PJ 1981 A role for skin in Ca metabolism of frog? Comp Biochem Physiol 68A:181–185

Pilkington JB, Simkiss K 1966 The mobilization of the calcium carbonate deposits in the endolymphatic sacs of metamorphosing frogs. J Exp Biol 45:329–341

Robertson DR 1971 Cytological and physiological activity of the ultimobrachial glands in the premetamorphic anuran, *Rana catesbeiana*. Gen Comp Endocrinol 16:329–341

Yamashita K, Koide Y, Itoh H, Kawada N, Kawauchi H 1995 The complete amino acid sequence of chum salmon stanniocalcin, a calcium regulating hormone in teleosts. Mol Cell Endocrinol 112:159–167

Robertson DR 1975 Effects of the ultimobranchial and parathyroid glands and vitamins D2, D3 and dihydrotachysterol2 on blood and intestinal calcium transport in the frog. Endocrinology 96:934–940

Robertson DR 1978 Diurnal variations in the influence of the ultimobrachial glands on calcium homeostasis in the frogs (*Rana pipiens*). J Endocrinol 79:167–178

Parmentier M, Ghysens M, Pypens F, Lawson DEM, Pasteels JL, Pochet R 1987 Calbindin in vertebrate classes: immunohistochemical localization and western blot analysis. Gen Comp Endocrinol 65:399–407

Das VK, Swarup K 1975 Effect of calcium-rich environment on the ultimobranchial glands of Rana tigrina. Arch Anat Microsc 64:261–272

Schlumberger HG, Burk DH 1953 Comparative study of the reaction to injury. III. Hypervitaminosis D in frog with special reference to lime sacs. Arch Pathol 56:103–124

Bruce HM, Parkes AS 1950 Rickets and osteoporosis in *Xenopus laevis*. J Endocrinol 7:67–81

Deleted in proof

Forman BM, Samuels HH 1990 Interactions among a subfamily of nuclear hormone receptor: the regulatory zipper model. Mol Endocrinol 4:1293–1301

Whitfield GK, Hsieh J-C, Nakajima S, MacDonald PN, Thompson PD, Jurutka PW, Haussler CA, Haussler MR 1995 A highly conserved region in the hormone-binding domain of the human vitamin D receptor contains residues vital for heterodimerization with retinoid X receptor and for transcriptional activation. Mol Endocrinol 9:1166–1179

Nakajima S, Hsieh J-C, MacDonald PN, Galligan MA, Haussler CA, Whitfield GK, Haussler MR 1994 The C-terminal region of the vitamin D receptor is essential to form a complex with a receptor auxiliary factor required for high affinity binding to the vitamin D-responsive element. Mol Endocrinol 8:159–172

Whitfield GK, Selznick SH, Haussler CA, Hsieh J-C, Galligan MA, Jurutka PW, Thompsom PD, Lee SM, Zerwekh JE, Haussler MR 1996 Vitamin D receptors from patients with resistance to 1,25-dihydroxyvitamin D3: point mutations confer reduced transactivation in response to ligand and impaired interaction with the retinoid X receptor heterodimeric partner. Mol Endocrinol 10:1617–1631

Kristjansson K, Rut AR, Hewison M, O'Riordan JLH, Hughes MR 1993 Two mutations in the hormone binding domain of the vitamin D receptor cause tissue resistance to 1,25-dihydroxyvitamin D3. J Clin Invest 92:12–16

Nakajima S, Hsieh J-C, Jurutka PW, Galligan MA, Haussler CA, Whitfield GK, Haussler MR 1996 Examination of the potential functional role of conserved cystein residues in the hormone-binding domain of the human 1,25-dihydroxyvitamin D3 receptor. J Biol Chem 271:5143–5149

Durston AJ, Timmermans JPM, Hage WJ, Hendriks HFJ, de Vries NJ, Heideveld M, Nieuwkoop PD 1989 Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 340:140–144

Sive HL, Draper BW, Harland RM, Weintraub H 1990 Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. Genes Dev 4:932–942

Kraft JC, Schuh T, Juchau M, Kimelman D 1994 The retinoid X receptor ligand, 9-cis-retinoic acid, is a potential regulator of early Xenopus development. Proc Natl Acad Sci USA 91:3067–3071

Minucci S, Saint-Jeannet J-P, Toyama R, Scita G, DeLuca LM, Taira M, Levin AA, Ozato K, Dawid IB 1996 Retinoid X receptor-selective ligands produce malformations in Xenopus embryos. Proc Natl Acad Sci USA 93:1803–1807

Achkar CC, Derguini F, Blumberg B, Langston A, Levin AA, Speck J, Evans RM, Bolado JJ, Nakanishi K, Buck J, Gudas LJ 1996 4-Oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors. Proc Natl Acad Sci USA 93:4879–4884

Ellinger-Ziegelbauer H, Dreyer C 1991 A retinoic acid receptor expressed in the early development of *Xenopus laevis*. Genes Dev 5:94–104

Blumberg B, Mangelsdorf DJ, Dyck JA, Bittner DA, Evans RM, De Robertis EM 1992 Multiple retinoid-responsive receptors in a single cell: families of retinoid "X" receptors and retinoic acid receptors in the Xenopus egg. Proc Natl Acad Sci USA 89:2321–2325

Yaoita Y, Brown DD 1990 A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. Genes Dev 4:1917–1924